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Minireview

Pathogenesis of renal ischemia/reperfusion injury: lessons from knockout mice

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Abstract

Ischemia/reperfusion-induced acute renal failure is a common clinical problem associated with a high morbidity and mortality. Upon hypoxic injury, the depletion of ATP causes mitochondrial dysfunction, and accumulation of intracellular sodium, calcium and reactive oxygen species. Subsequently, multiple enzyme systems including proteases, nitric oxide synthases, phospholipases and endonuclease are activated and responsible for cytoskeleton disruption, membrane damage, and DNA degradation, and eventually cell death. Ischemia/reperfusion injury also activates complement, cytokines, and chemokines, which are cytotoxic themselves, but also attract leukocytes into the ischemic area to cause further damage. The vascular endothelial cell injury and dysfunction prolong ischemia and induce vascular congestion, edema, and further infiltration of inflammatory cells. Many players in renal ischemia/reperfusion injury and their mechanisms have been investigated using genetically manipulated mouse models. In this review, we focus on the information gathered from these studies. Deficiency of the Na/Ca exchanger, inducible nitric oxide synthase, Caspase-1, A3 adenosine receptor, C3, C5, C6, Factor B, or medkine protects the kidney against I/R injury. Conversely, deficiency of the interleukin-1 receptor, osteopontin, C4, or recombination activation gene-1 is not protective, while the absence of adrenomedullin or endothelin receptor B delays the recovery of ischemia/reperfusion injury. The knowledge obtained from these studies provides new direction for designing potential therapeutic agents for treating ischemia/reperfusion injury.

Keywords: Ischemia; Hypoxia; Reperfusion; Intracellular calcium; Renal proximal tubular cells; Knockout mice

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Introduction

Ischemia/reperfusion- (I/R) induced acute renal failure is associated with a high morbidity and mortality rate in hospitalized patients. The mechanisms underlying renal I/R injury are complex. These have been subjects of several review articles, however, many steps are still unclear (Thadhani et al., 1996; Edelstein et al., 1997; Sutton and Molitaris, 1998; Sheridan and Bonventre, 2001; Molitoris, 1998; Padanilam, 2003). Recently, genetically manipulated animal models such as transgenic and knockout (KO) mice with enhancement and absence, respectively, of certain proteins, have been developed and used for searching the mechanisms of renal I/R injury. These models have certain limitations; for example, KO mice may develop compensatory mechanisms, that may alter the susceptibility to I/R injury, or mask the effect of depletion of the targeted protein. In addition, the deletion of certain genes may be lethal, therefore, no KO mice can be used to test the role of respective proteins in I/R injury. Nevertheless, the studies using KO mouse models in general provide important insight into I/R injury as well as the information that otherwise is not available from in vivo studies using normal animals. In this article, we review the results of studies from the literature using KO mice as a model to investigate mechanisms of renal I/R injury (Table 1).

Table 1
Renal ischemia/reperfusion injury in knockout mice

Molecule	Genotype	Protection	Implication	Reference
NCX1	Heterozygote	Yes	↓ [Ca]i ↓ ET-1	Yamashita et al., 2003
Caspase-1	Homozygote	Yes	↓ IL-18 ↓ neutrophil infiltration	Melnikov et al., 2001
IL-1R	Homozygote	No	I/R injury independent of IL-1	Haq et al., 1998
Midkine	Homozygote	Yes	↓ leukocyte infiltration	Sato et al., 2002
Osteopontin	Homozygote	No	↓ macrophage infiltration ↓ interstitial fibrosis ↑ apoptosis	Persy et al., 2003
iNOS	Homozygote	Yes	↑ HSP72	Ling et al., 1999
AM	Heterozygote	No	↓ NO release Vasoconstriction	Nishimatsu et al., 2002
C3,C5,C6	Homozygote	Yes	↓ neutrophil infiltration ↓ MAC activity	Zhou et al., 2000
C4	Homozygote	No	I/R injury independent of Classic pathway	Zhou et al., 2000
Factor B	Homozygote	Yes	↓ C3 deposition ↓ neutrophil infiltration	Thurman et al., 2003
RAG-1	Homozygote	No	I/R injury independent of B and T cells	Park et al., 2002
ICAM-1	Homozygote	Yes	↓ leukocyte adhesion ↓ leukocyte infiltration	Kelly et al., 1996
ET_BR	Rescued Homozygote#	No	Worsen recovery phase ↑ ET-1	Nishida et al., 2002
A3AR	Homozygote	Yes	Anti-apoptotic?	Lee et al., 2003

NCX1: Na, Ca exchanger-1; IL-1R: interleukin-1 receptor; iNOS: inducible nitric oxide synthase; AM: Adrenomedullin; RAG-1: recombination activation gene-1; ICAM-1: intercellular adhesion molecule-1; ET_BR: endothelin B receptor; A3AR: A3 adenosine receptor; I/R: ischemia/reperfusion; ET-1: endothelin-1; HSP: heat shock protein; MAC: membrane attack complex; #Rats with naturally occurring ET_BR deficiency which are rescued (see text).

Upon I/R insult, the depletion of energy in renal epithelial cells activates many systems, that directly cause disruption of the cytoskeleton and cell polarity, and cell death, or indirectly, via activating other cellular systems such as endothelial cells and leukocytes, resulting in tubulointerstitial damage. Both necrosis and apoptosis occur after I/R injury. It appears that a severe depletion of ATP favors necrotic cell death whereas GTP depletion tends to promote apoptotic cell death (Padanilam, 2003). Vascular endothelial cell injury and dysfunction induce vascular congestion and edema, diminish blood flow, and stimulate infiltration of inflammatory cells such as neutrophils (Sutton et al., 2002). Activated neutrophils can release cytokines, reactive oxygen species, proteases, myeloperoxidase and other enzymes to trigger further damage (Lauriat and Linas, 1998). At the same time, certain defense mechanisms are also activated, such as induction of heat shock protein (HSP) in order to protect cells against damage (Smoyer et al., 2000), or growth factors to stimulate regeneration of tubules (Hammerman, 1998). Therefore, ischemic preconditioning protects the kidney against the subsequent I/R injury (Bonventre, 2002). In the following sections, we briefly review each major system involved in renal I/R injury and discuss the contribution of results obtained from KO mice to the overall mechanisms of renal I/R injury.

Intracellular free calcium

When cells are subjected to hypoxic insult, the depletion of ATP shuts down Na, K-ATPase activity. Subsequently, [Na]i increases and both the Na,H exchanger and Na/Ca exchanger (NCX) are probably activated in order to pump out Na. The consequence is that intracellular pH decreases and intracellular Ca increases. Intracellular calcium is one of the key mediators of hypoxic injury in renal epithelial cells. Intracellular free calcium activates proteases, phospholipases, nitric oxide synthases (NOS) and endonucleases which in turn break down the cytoskeleton, damage cellular proteins and membrane and degrade DNA (Thadhani et al., 1996; Edelstein et al., 1997; Padanilam, 2003). In cultured renal proximal tubular cells, hypoxia-induced cellular damage can be ameliorated by calcium channel blockers, intracellular calcium chelator and calcium binding protein, calbindinD28k (Almeida et al., 1992; Kribben et al., 1994; Wu et al., 2002).

NCX1 KO mice

Yamashita and coworkers (see Yamashita et al., 2003) used heterozygote NCX1 KO mice to prove the role of NCX1 in renal I/R injury. The heterozygous mice were used because homozygotes died of heart failure before birth. The renal NCX1 protein expression in the NCX1 +/- mice is about half of that in the wildtype. The KO mice are more resistant to I/R injury. In addition, they have much lower renal endothelin-1 (ET-1) content after I/R compared with wildtype. This study confirms again the role of intracellular Ca in renal I/R injury, and indicates that the NCX1 mediates accumulation of intracellular Ca and possibly the production of ET-1.

Protease system

Two groups of cysteine proteases, calpains and caspases, have been shown to play an important role in renal I/R injury. Calpains are calcium-dependent cysteine proteases which are activated by elevated

[Ca]i during I/R injury. Caspases are a family of proteases which have two major functions: to promote apoptotic cell death (caspase-2, -8, -9, and -10 are initiators, and caspase-3, -6 and -7 are effectors) and to activate proinflammatory cytokines (caspase-1, -4 and -5). Kaushal et al have shown that caspase -1, -2 and -3 are upregulated in kidneys after I/R injury (Kaushal et al., 1998). Interestingly, there is an interaction between calpains and caspases during I/R injury. The level of calpain inhibitor, calpastatin, is markedly diminished during I/R injury. This decrease can be prevented by prior treatment of caspase inhibitors. Therefore, it is likely that caspases may enhance calpain activity by degrading its intrinsic inhibitor (Shi et al., 2000).

Caspase-1 KO mice

Caspase-1 is a proinflammatory protease, which mediates apoptosis and activates cytokines such as interleukin (IL)-1 and IL-18. Melnikov and colleagues (Melnikov et al., 2001) have demonstrated that caspase-1 KO mice are more resistant to I/R injury compared to wildtype mice. Not only does tubular necrosis score decrease, neutrophil infiltration also decreases. Unlike the wildtype mice, which have a marked increase in the renal IL-18 level, the KO mice do no have elevated IL-18. Interestingly, the absence of caspase-1 does not prevent I/R-induced apoptosis. These findings suggest that caspase-1 plays an important role in neutrophil-mediated cellular damage via its activation of IL-18. The lack of protective effects of caspase-1 KO against I/R-induced apoptosis confirms that caspase-1 is not involved in apoptotic process in renal cells.

Cytokines

Cytokines are released from the damaged organ and stimulate adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), and chemokines, such as macrophage inflammatory protein 2 (MIP-2) and monocyte chemoattractant protein 1 (MCP-1). These molecules trigger infiltration of inflammatory cells into the tubulointerstitium and cause further damage. Several cytokines such as TNF-α, IL-1, and IL-18 have been implicated in the renal I/R injury. In addition, there are newly identified cytokines in the kidney such as midkine (MK) and osteopontin, whose role in I/R injury has not been defined. Three KO mouse studies are discussed below.

IL-1 receptor KO mice

Haq and coworkers (Haq et al., 1998) have demonstrated that the absence of IL-1 receptor (IL-1R) does not protect against renal I/R injury in the KO mice. These findings are consistent with the lack of protection by IL-1R antagonist against renal I/R injury.

Midkine KO mice

Midkine (MK) is a newly identified multifunctional heparin-binding growth factor and cytokine (Takada et al., 1997). MK promotes migration of neutrophils and macrophages but is weakly expressed in the proximal tubules. After I/R, MK is upregulated in the proximal tubules, and induced in distal tubules. The absence of MK is protective against renal I/R injury by reducing leukocyte infiltration.

Furthermore, the upregulation of chemokines such as MIP-2 and MCP-1 by I/R injury is diminished in the MK KO mice (Sato et al., 2002). These results indicate that MK is produced locally and mediates I/R-induced tubulointerstitial damage as a cytokine.

Osteopontin KO mice

Osteopontin is a phosphoprotein that has multiple functions including serving as a macrophage chemoattractant and a survival factor for tubular cells. It is upregulated after renal I/R injury, but its role is not clear. The absence of osteopontin does not change the acute course of renal I/R injury within the first 7 days. Osteopontin KO mice had enhanced apoptosis, but reduced macrophage infiltration and interstitial fibrosis. Therefore, the lack of protection is probably due to canceling out the beneficial effects (reduced macrophage infiltration) by the deleterious effect (enhanced apoptosis) (Persy et al., 2003).

NO synthase

The role of NO on I/R injury has been controversial because of the complexity of NO synthase (NOS) isoforms (Goligorsky et al., 2002). NOS activity is increased during hypoxia in isolated renal proximal tubules and the NOS inhibitor, L-NAME, is cytoprotective in this system (Yu et al., 1994). Ling (Ling et al., 1998) further showed that only proximal tubules isolated from inducible NOS (iNOS) KO mice were resistant to hypoxic injury, whereas endothelial NOS (eNOS) KO or neuronal NOS (nNOS) KO were not. Furthermore, Noiri and colleagues (Noiri et al., 1996) have shown that in vivo targeting of iNOS with antisense oligonucleotides protects the rat kidney against ischemia. On the other hand, Linas and coworkers (Linas et al., 1997) have shown that NO blocks leukocyte infiltration and the deleterious effects of activated leukocytes on renal function. There are two KO mouse studies related to the NOS system.

iNOS KO mice

The absence of iNOS attenuates renal I/R injury both functionally and morphologically. However, the role of iNOS in I/R injury is unclear because there is no iNOS induction after I/R injury in the wildtype mice. Instead, the protective effect is probably due to the unexpected upregulation of inducible HSP72 in the KO mice under basal condition (Ling et al., 1999). In other words, iNOS KO mice are similar to animals with ischemic preconditioning, in which HSP is activated by prior ischemic insults (Park et al., 2001).

Adrenomedullin KO mice

Adrenomedullin (AM) is a potent depressor peptide, which releases NO. After I/R injury, AM heterozygote KO mice (homozygote is lethal) had more severe renal damage with higher blood urea nitrogen levels and renal damage scores. On the other hand, AM transgenic mice, which express a higher level of AM, are protected from I/R with a higher renal NOS activity. Therefore, AM plays a role in regulation of vascular tone and in renoprotection against ischemia through its NO releasing activity

(Nishimatsu et al., 2002). Taking the results from iNOS and AM KO mice together, it appears that eNOS, which is stimulated by AM, may play a protective role in renal I/R injury, while the role of iNOS remains to be proven.

Complement system

The complement system has been implicated in the pathogenesis of I/R injury. Deposition of C3 occurs primarily at the tubular basement membrane in the area of tubular damage (Zhou et al., 2000). Complement activation can cause endothelial activation, neutrophil infiltration, vasoconstriction, and direct cellular damage by the membrane attack complex (MAC).

C3, C4, C5, C6 KO mice

To determine the relative importance of the early (C3, C4), intermediate (C5), and late (C6) components of the complement cascade, each KO mice were used in renal I/R injury experiments (Zhou et al., 2000). This elegant study provides critical information which is not available elsewhere. First, the C3-deficient mice are protected from renal I/R injury, whereas C4-deficient mice are not protected. Since C3 is involved in both classic and alternative pathways, and C4 is only active in the classic pathway, this finding indicates that complement activation during I/R injury occurs via the alternative pathway. Secondly, neutrophil infiltration is reduced equally in C3-, and C6-deficient mice. Since C3-deficient mice do not produce C3a and C5a, while C6-deficient mice have normal levels of C3a and C5a, this finding minimizes the importance of C3a and C5a in the chemotaxis of renal I/R injury. Finally, the finding that C6-deficiency alone provides significant protection suggests that MAC, which contains C5 and C6, is likely to be responsible to the complement-mediated tubular damage.

Factor B KO mice

Factor B is an essential component of the alternative complement activation pathway. Factor B KO mice developed substantially less functional and morphologic renal injury after I/R and no C3 deposits or neutrophil infiltration in the outer medulla (Thurman et al., 2003). This study confirms the importance of the alternative pathway in renal I/R injury and suggests that blocking alternative pathway may be a useful therapeutic approach for treating ischemic renal failure.

Recombination-activation gene-1 KO mice

Recombination-activation gene (RAG)-1 mediates maturation of B and T cells. The classic pathway of complement activation is silent in RAG-1 KO mice due to the absence of Ig. When subjected to I/R renal injury, RAG-1 KO mice are not protected. Complement deposition still occurs in these KO mice indicating that the alternative pathway is responsible for the damage. It appears that renal I/R injury is also independent of T cells (Park et al., 2002). Taking all these results from KO mice, it is clear that complement activation in the kidney after I/R occurs exclusively via the alternative pathway. The MAC may account for the effect of complement on

renal I/R injury in the epithelial cells, while C3a and C5a may not have a significant role in neutrophil infiltration.

Leukocyte adhesion molecules

Leukocyte adhesion is a central event in leukocyte recruitment to injured tissue. It is mediated by leukocyte adhesion molecules including Selectins, Mucins, Integrins and Ig-like adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Adler and Brady, 1999). The adhesion of leukocytes to endothelial cells promotes dynamic cross-talk between the two cell types that facilitates leukocyte infiltration and activation, free radical generation, generation of proinflammatory mediators and tubulointerstitial damage.

ICAM-1 KO mice

This is the first KO mouse model used to investigate the pathogenesis of renal I/R injury. ICAM-1 is upregulated after renal I/R injury probably due to high circulatory TNF-α and IL-1 levels. The absence of ICAM-1 protects the kidney by reducing leukocyte infiltration. Similar protection can be achieved by neutrophil depletion (Kelly et al., 1996). These results strongly support the role of ICAM-1 and leukocytes in renal I/R injury.

Endothelin system

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide produced mainly by endothelial cells, but also by smooth muscle cells and renal tubular epithelial cells. ET-1 acts through activation of G protein-coupled ET_A and ET_B receptors (Rubanyi and Polokoff, 1994). ET_A receptor is present primarily in the vascular tissues, while ET_B receptor is distributed abundantly in the collecting tubules. ET-1 is upregulated after renal I/R injury and may contribute to the injury by prolonging vasoconstriction. It is known that ET_A-selective, but not ET_B-selective, antagonist is protective against renal I/R injury (Kuro et al., 2000). In order to define the role of ET_B in I/R renal injury better, a unique rat model of ET_B deficiency has been studied.

ET_B receptor-deficient rats

Spotting-lethal rat carries a naturally occurring deletion in the ET_B receptor gene. Genetic rescue by using a dopamine-beta-hydroxylase-ET_B transgene produces ET_B -deficient adult rats, which express ET_B receptor in the adrenal glands and other adrenergic neurons, but not in other tissues including the kidney. Their ET_A receptor is not affected. Rescued homozygous rats did not have protective effects after I/R. In fact, deficient rats had a delayed recovery and higher renal ET-1 content comparing to wild type rats. It is possible that activation of ET_B is renoprotective by increasing clearance of ET-1, and/or via other actions such as stimulating mitogenesis or medullary vasodilatation (Nishida et al., 2002). Therefore, the ET receptor specificity is critical in drug development of ET-1 antagonists for treating I/R renal injury.

Adenosine system

Adenosine is a vasoactive hormone. Its action is mediated through 4 receptors; all of them are present in the kidney (Zou et al., 1999). Activation of the A1 receptor promotes vasoconstriction as well as tubular sodium reabsorption, while activation of A2a and A2b promotes vasodilation. Adenosine is released in large amounts after ischemia (Osswald et al., 1977). Activation of A2a is protective against renal I/R injury by reducing neutrophils infiltration, and suppression of P-selectin and ICAM-1 (Okusa et al., 2000). The function of the A3 receptor in the kidney is, however, unclear.

A3 adenosine receptor KO

The absence of A3 receptor is protective against renal I/R injury both functionally and morphologically. However, the protective mechanisms of A3 receptor KO are not clear. One possibility is that the absence of A3 receptor may be anti-apoptotic because A3 activation has been shown to promote apoptosis in several cell lines including renal cells (Lee et al., 2003).

Conclusion

Information obtained from renal I/R injury experiments in KO mice is valuable. For example, the complement KO mice provide a clear conclusion that the alternative pathway, rather than classic pathway, is involved in renal I/R cellular damage. The results from caspase-1 KO mice emphasize the role of IL-18 in renal I/R injury, while results from IL-1R KO mice rule out the involvement of IL-1. This approach also complements the pharmacological studies in which antagonists or agonists for specific receptor blockers are tested. Knowledge obtained from these studies will provide directions for developing new therapeutic agents for renal I/R injury.

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ARTICLE

Distribution of Group II Phospholipase A2 Protein and mRNA in Rat Tissues

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SUMMARY Group II phospholipase A2 (PLA2) is an acute-phase protein and an important component of the host defense against bacteria. In this study we investigated the distribution of PLA2 protein by immunohistochemistry and the distribution of mRNA of PLA2 by Northern blotting and in situ hybridization in rat tissues. PLA2 protein was localized in the Paneth cells of the intestinal mucosa, chondrocytes and the matrix of cartilage, and megakaryocytes in the spleen. By Northern blotting, mRNA of PLA2 was found in the gastrointestinal tract, lung, heart, and spleen. By in situ hybridization, PLA2 mRNA was localized in the Paneth cells of the small intestinal mucosa but in no other cell types. Our results show specific distribution of PLA2 in a limited number of cell types in rat tissues. The reagents developed in this study (the anti-rat PLA2 antibody and probes for Northern blotting and in situ hybridization of mRNA of rat PLA2) will provide useful tools for future studies concerning the role of PLA2 in various experimental disease models.

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KEY WORDS
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phospholipase A2
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recombinant protein

PHOSPHOLIPASE A2 plays an important role in inflammation as a rate-limiting enzyme in the production of proinflammatory mediators derived from arachidonic acid (Vadas et al. 1993). A number of different low molecular weight secretory phospholipase A2 enzymes are present in mammalian tissues (Cupillard et al. 1997; Dennis 1997; Tischfield 1997). Group II phospholipase A2 (PLA2) was originally purified from synovial fluid and platelets (Kramer et al. 1989; Seilhamer et al. 1989). In humans, PLA2 has been also found in the Paneth cells of the small intestine and in a wide range of other tissues (Kiyohara et al. 1992; Nevalainen and Haapanen 1993; Kallajoki and Nevalainen 1997). PLA2 is an acute-phase protein (Crowl et al. 1991). Increased concentrations of PLA2 have been found in serum in various inflammatory diseases, including sepsis, infections, and acute pancreatitis (Nevalainen and Grönroos 1997). Mobilization of PLA2 during inflammation may play an important role in the host defense against invading Gram-positive bacteria (Harwig et al. 1995; Weinrauch et al. 1996,1998; Foreman-Wykert et al. 1999; Laine et al. 1999). Although there is evidence that PLA2 in blood plasma may originate from the liver in humans under some circumstances (Nevalainen et al. 1996), the source of circulating PLA2 in inflammatory diseases is still unknown.

The gene coding for rat Group II phospholipase A2 was cloned from the spleen (Ishizaki et al. 1989), platelets (Komada et al. 1990), liver (Van Schaik et al. 1993), and heart (De Windt et al. 1997). Southern blotting analysis of genomic DNA revealed that there is only a single copy in the rat genome (Komada et al. 1990). There are minimal differences between the reported cDNA sequences, obviously due to the study of different rat strains. The amino acid sequences of PLA2s from the spleen, platelets, and heart are identical, and in PLA2 from the liver there is only one amino acid difference compared to the PLA2s from other sources. The coding sequence of the rat PLA2 is 758 nucleotides, which predicts a 146-amino-acid protein with high homology (72%) to human PLA2 (Ishizaki et al. 1989). The rat PLA2 contains a 21-amino-acid N-terminal signal sequence, 14 cysteine

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residues, and a six-amino-acid C-terminal extension, which are characteristic of Group IIA phospholipase A2 (Tischfield 1997).

The purpose of this study was to investigate the distribution of PLA2 protein and mRNA in rat tissues. We produced recombinant rat PLA2 and polyclonal antibodies against it, and localized the PLA2 protein by immunohistochemistry and the mRNA of PLA2 by Northern and in situ hybridizations. Our results show that PLA2 is expressed in few specific cell types. The strongest expression of PLA2 is found in the Paneth cells of the intestinal mucosa.

Materials and Methods

Reagents

Rat Group II phospholipase A2 cDNA sequence cloned in the EcoRI and XbaI sites of pGEM4 (Promega; Madison, WI) was obtained from Shionogi Research Laboratories (Osaka, Japan). The bacterial expression vector pQE-60 was purchased from QIAGEN (Chatsworth, CA). Restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) except for BspHI, which was from New England Biolabs (Beverly, MA). DNA ligase was from Life Technologies, Gibco BRL (Paisley, UK). Primers were synthesized at the University of Kuopio (Kuopio, Finland). *E. coli* JM109 cells were obtained from Department of Microbiology (University of Turku, Turku, Finland) and M15 and SG13009 cells from QIAGEN. Unless otherwise noted, all other reagents were from Sigma (St Louis, MO).

PCR Amplification

Three oligonucleotide primers of sense and antisense orientations based on the sequence of rat PLA2 cDNA were synthesized. The forward primer sequences were (412) 5′-ATA TCC ATG GAT GAA GGT CCT CCT GTT GC-′3 and (551) 5′-ATA TTC ATG AGC CTT CTG GAG TTT GGG-′3 and the reverse sequence (413) 5′-ATA AGA TCT GCA ACT GGG CGT CTT CCC-′3. The region of rat PLA2 cDNA that encodes the signal peptide and the mature protein was amplified by PCR. Primer 412 has a unique NcoI site. Biotinylated primer 551 contained the sequence for BspHI and the initiating methionine. Primer 413 has a unique BglII site. Rat PLA2 contains the BspHI site in the protein coding sequence starting at nucleotide 258. PCR was carried out in 100-µl reaction volume containing 100 mM Tris-HCl (pH 8.3), 0.2 mM dNTP, 1 µM antisense primer, 1 µM sense primer, and 2.5 U Taq DNA polymerase (Finnzymes; Espoo, Finland). Conditions for 35 cycles of PCR were 95C/30 sec, 58C/60 sec, and 72C/45 sec.

Cloning and Expression of Rat Group II Phospholipase A2

Cloning of rat PLA2 was carried out in two steps (Figure 1). The first PCR was done with primers 412 and 413. The product was extracted from the gel and digested with NcoI and BglII. The digested product was ligated to pQE-60 vector previously digested with NcoI and BglII. This construct (pQE-60-PLA2-sham), which is not in frame, was used as the first step in cloning and later as a negative control. The second step was PCR with primers 551 and 413. The PCR product was attached to streptavidin-coated magnetic beads (Boehringer Mannheim). The product was digested with BamHI, washed, digested with BspHI, and recovered. The product was ligated and cloned to the pQE-60-PLA2-sham vector previously digested with NcoI and BamHI, resulting in the plasmid pQE-60-PLA2. Finally, successful construction was confirmed by sequencing.

The plasmid pQE-60-PLA2 was transformed into *E. coli* strain JM 109. Transformants were selected on LB–agar plates supplemented with 100 µg/ml ampicillin. For induction of gene expression, *E. coli* JM109 cells containing pQE-60-PLA2 were grown at 30C in LB medium containing 100 µg/ml ampicillin. After reaching an $OD_{550} \approx 1.0$, isopropyl-β-D-thio-galactoside (IPTG) was added to a final concentration of 1 mM. The cells were harvested by centrifugation and stored as pellets at −70C.

Preparation of Recombinant Rat Group II Phospholipase A2

Recombinant PLA2 was found exclusively in the inclusion bodies of *E. coli*. A cell pellet from 300 ml of bacterial culture (1.5 g wet weight) was thoroughly washed with buffer containing 100 mM Tris-HCl (pH 7.3) and 150 mM NaCl. The cells were lysed with an ultrasound sonicator (Labsonic L; Braun Diessel Biotech, Melsungen, Germany) at maximal

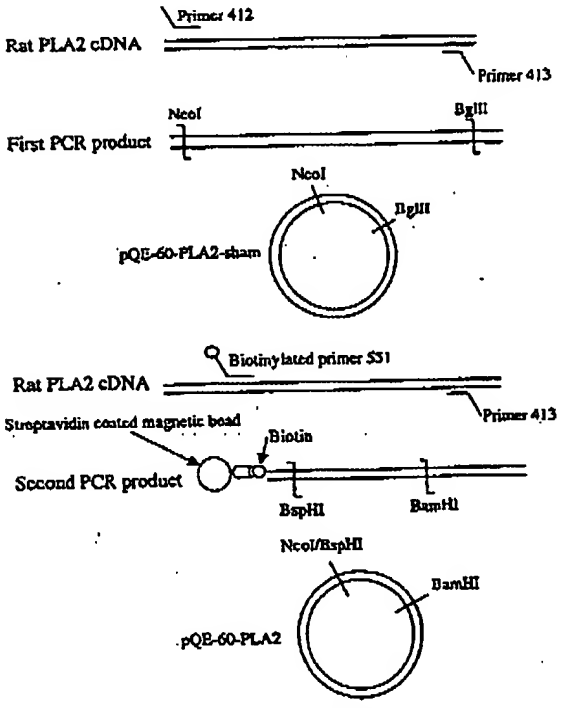

Figure 1 Cloning of rat Group II phospholipase A2.

power twice for 30 sec. Lysozyme was added to a final concentration of 1 mg/ml, incubated for 30 min at room temperature (RT) and sonicated. The pellet was harvested by centrifugation at 10,000 × g for 10 min and resuspended in 20 ml buffer containing 6 M guanidine hydrochloride and 100 mM Tris-HCl (pH 7.3). After incubation overnight at RT, silica was added to a final concentration of 1% and the cell lysate was pelleted by centrifugation. The supernatant was recovered and β-mercaptoethanol was added to a final concentration of 0.2%. The recombinant PLA2 was further purified by using nickel affinity chromatography. Briefly, a 20-ml sample was loaded onto an affinity column charged with Ni^{2+} and washed with 6 M guanidine hydrochloride, pH 7.6 and pH 5.3, and PLA2 was eluted with 6 M guanidine hydrochloride, pH 4.1. The eluate was dialyzed against water overnight at 4C. Amino-acid sequence analysis was performed with an Applied Biosystems (Foster City, CA) model 477A protein sequencer equipped with an on-line Applied Biosystems model 120A phenylthiohydantoin amino acid analyzer. The sample for mass spectrometry was mixed with sinapinic acid matrix and analyzed by a matrix-assisted laser desorption/ionization mass spectrometer (MALDI-MS; Lasermat, Finnigan Mat, Bremen, Germany).

Measurement of the Catalytic Activity of Phospholipase A2

The catalytic activity of phospholipase A2 was measured by a modified radiometric method (Schädlich et al. 1987) with L-α-1-palmitoyl-2-arachidonyl-containing phosphatidyl ethanolamine (NEN Life Science Products; Boston, MA) as a substrate.

Preparation of Antiserum

Three New Zealand White rabbits were immunized SC with 250 μg of purified recombinant rat PLA2 in Freund's complete adjuvant. Four booster injections of 200 μg of PLA2 in Freund's incomplete adjuvant were administered after 2, 4, 6, and 8 weeks. Blood was collected from each rabbit before each booster injection.

Western Blotting

Purified recombinant rat PLA2 and ileal lysates from Sprague–Dawley rats were separated in 4–15% SDS-PAGE gels (Phastsystem; Pharmacia, Uppsala, Sweden) and transferred to nitrocellulose filters (Schleicher & Schuell; Dassel, Germany). Anti-rat PLA2 antiserum diluted 1:5000 was used as the primary antibody. The immunoreaction was detected with a Vectastain avidin–biotin–peroxidase complex (ABC) kit (Vector Laboratories; Burlingame, CA) according to the manufacturer's instructions.

Immunohistochemistry

Tissues from various rat organs were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections were incubated with several different dilutions ranging from 1:5000 to 1:20,000 of anti-rat PLA2 antiserum with 0.15 M NaCl in 0.05 M Tris buffer, pH 8.6, containing 1% bovine serum albumin. Rabbit anti-human von Willebrand factor (DAKO; Glostrup, Denmark) was diluted 1:1000. The immunoreaction was localized with a Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. For control staining, the primary antibody was replaced by preimmune rabbit serum. The sections were counterstained with hematoxylin.

Northern Hybridization

RNA was isolated by the guanidine isothiocyanate/acid phenol method (Chomzynski and Sacchi 1987). Ten μg of total RNA was electrophoresed through a 1% agarose/formaldehyde gel and transferred to GeneScreen nitrocellulose membrane (NEN Life Science Products) with 10 × SSPE overnight. The RNA was crosslinked to the filter by UV irradiation and the filter was baked at 80C for 1 hr. A 10 × 15-cm filter was prehybridized for 4 hr at 42C in 7.5 ml Presoak (50% deionized formamide, 4 × SSPE, 5 × Denhardts solution, 1% SDS, 100 μg/ml denatured ssDNA) in a rotary incubator (Hybaid; Middlesex, UK). The prehybridization solution was replaced by 7.5 ml fresh Presoak containing the denatured probe, ^{32}P-labeled EcoRI/XbaI fragment of PLA2/pGEM4 (Random Prime Labelling Kit; Boehringer Mannheim). The hybridization was performed in a rotary incubator at 42C overnight. The filter was washed twice for 15 min in 2 × SSPE/0.1% SDS at RT, 15 min in 2 × SSPE/0.1% SDS at 42C, 15 min in 0.1 × SSPE/0.1% SDS at RT, 15 min in 0.1 × SSPE/0.1% SDS at 42C, 15 min in 0.1 × SSPE/0.1% SDS at 56C, and 15 min in 0.1 × SSPE/0.1% SDS at 65C. The filter was exposed to Hyperfilm MP (Amersham; Poole, UK) with intensifying screens at −70C for 7 days. Several different exposures varying from 3 hr to 7 days were taken.

In Situ Hybridization (ISH)

ISH was performed on sections of formalin-fixed, paraffin-embedded tissues as described previously (Nevalainen et al. 1998), with slight modifications. Briefly, the 0.75-kb rat PLA2 cDNA insert was cloned into the XbaI and EcoRI sites of pGEM-4 vector (Ishizaki et al. 1989). Digoxigenin-labeled cRNA probes were synthesized by in vitro transcription with T7 (sense) and SP6 (antisense) RNA polymerases, and the yields were estimated by using commercial kits (DIG RNA Labelling Kit and DIG Nucleic Acid Detection Kit; Boehringer Mannheim) as described in the kit protocols. Immediately before hybridization, the sections were rehydrated in PBS, treated with ficin (Zymed Digest-All; Zymed Laboratories, San Francisco, CA) for 10 min at 37C, washed in PBS, pH 7.4, and 2 × SSC (standard saline citrate; 1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5 min each. The hybridization solution contained either the antisense or sense digoxigenin-labeled cRNA probe at a final concentration of 5 ng/ml. Hybridization was performed overnight at 42C in a humidified chamber. After hybridization, the slides were rinsed in 2 × SSC and washed in 2 × SSC for 5 min at RT, three times for 5 min in 60% formamide at 60C, and twice for 5 min in 2 × SSC at RT. For the detection of digoxigenin-labeled cRNA probes, all incubations were performed at RT in a humidified lightproof chamber. The tissue sections were washed, blocked, and treated with a 1:2000 dilution of

alkaline phosphatase-labeled anti-digoxigenin Fab fragments (Boehringer Mannheim). The slides were washed in TBS (100 mM Tris-HCl, 100 mM NaCl, pH 7.5) containing 0.05% Triton X-100 twice for 5 min and in TBS for 5 min, and rinsed in the development buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The tissue sections were covered with 500 µl substrate solution consisting of 0.18 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Boehringer Mannheim), 0.34 mg/ml 4-nitroblue tetrazolium chloride (NBT) (Boehringer Mannheim), and 0.24 mg/ml levamisole (Vector Laboratories) dissolved in the development buffer. The color reaction was allowed to develop for 10 hr at 4C and was stopped by washing twice for 5 min in 10 mM Tris, pH 8.0, containing 1 mM EDTA. The sections were counterstained with hematoxylin.

Results

Cloning and Expression of Rat Group II Phospholipase A2

PCR was used to amplify the protein coding sequence from rat PLA2 cDNA. The cDNA contains a BspHI site in the protein coding sequence at nucleotide 258. To avoid addition of extra amino acids to the N-terminus of the recombinant protein, PCR was done in two steps as described in Materials and Methods. A 378-bp DNA product was obtained (Figure 2). The DNA product was inserted into NcoI/BspHI and BglII sites of the pQE-60 expression vector to produce pQE-60-PLA2. This expression system produces a recombinant protein with six additional adjacent histidine residues at the C-terminus of the inserted gene. Plasmids were transformed into JM109 *E. coli* and selected bacteria were submitted to induction protocols.

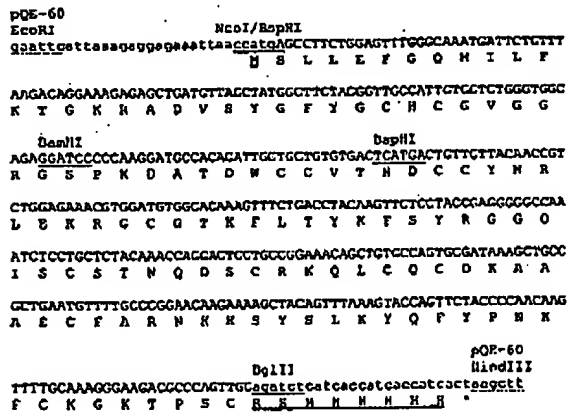

Figure 2 Expression construct pQE-60-PLA2. Restriction enzyme sites used in the cloning are underlined. NcoI/BspHI is the ligation of NcoI (C'CATG_G) to BspHI (T'CATG_A). The partial pQE-60 vector sequence from EcoRI to HindIII (sites marked with dotted underlining) is in lower case. Amino acids not present in native rat Group II phospholipase A2 are marked with wavy underlining.

After the induction of plasmid pQE-60-PLA2 with IPTG, significant production of rat Group II phospholipase A2 recombinant protein could be achieved in JM109 *E. coli*, as visualized by the appearance of a protein band of approximately 18 kD in SDS-PAGE (Figure 3). We also found duplicates of recombinant PLA2 when more protein was loaded in SDS-PAGE (Figure 3). Recombinant PLA2 was found exclusively in the inclusion bodies of JM109 *E. coli*, whereas no recombinant protein was found in the soluble fraction of JM109 *E. coli*. The maximal rate of production of the recombinant protein was reached at 12 hr after the induction. For practical purposes, production was allowed to continue overnight. The induction of plasmid pQE-60-PLA2 was also tested in strains M15 and SG13009 of *E. coli*, but no production of the recombinant protein was seen in these bacteria.

Large-scale Expression and Purification of Recombinant Rat Group II Phospholipase A2

JM109 *E. coli* harboring the plasmid pQE-60-PLA2 were grown in 1 liter of LB medium and induced with isopropyl-β-D-thio-galactoside (IPTG). When bacterial cells were ruptured by sonication under nondenaturing conditions, all recombinant rat PLA2 protein was recovered in the insoluble pellet. The recombinant protein was purified by nickel affinity chromatography under denaturing conditions. Typically, yields of approximately 40 mg of recombinant protein per 1 liter of culture, with a purity of ≥97%, were achieved. Sequencing the first six amino acids of the product revealed that the initiator methionine was uncleaved in 17% of the recombinant proteins. In 83% of the protein, the first amino acid was serine as in the native protein (Ono et al. 1988). Recombinant rat PLA2 was generated for the production of antibodies, and therefore no

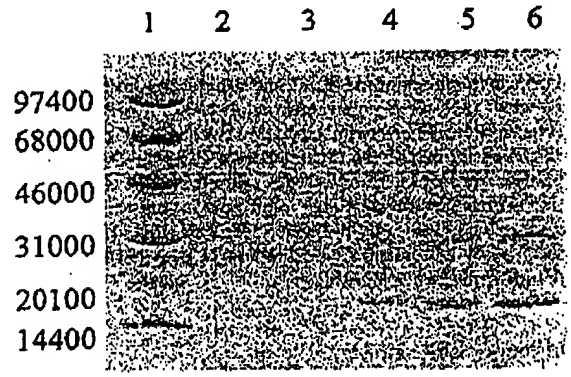

Figure 3 SDS-PAGE of recombinant rat Group II phospholipase A2. Lane 1, molecular weight markers; Lane 2, 10 ng; Lane 3, 50 ng; Lane 4, 100 ng; Lane 5, 200 ng; and Lane 6, 400 ng of recombinant PLA2.

attempt was made to refold the protein. The recombinant PLA2 showed a catalytic activity of approximately 1000 U/mg.

Production of Antibody

Once a sufficient amount of recombinant protein was available, antiserum was raised in rabbits against rat PLA2. To verify that the antibody produced to the recombinant protein would recognize native rat Group II phospholipase A2, an extract of rat ileum was analyzed by Western blotting. The antibody recognized a 14-kD protein (Figure 4). The antibody was used to detect the distribution of PLA2 protein in rat tissues by immunohistochemistry.

Crossreactivity of Anti-rat Group II Phospholipase A2 Antibody

Crossreactivity of the antibody was studied with sections of formalin-fixed, paraffin-embedded samples of human small intestine (Nevalainen et al. 1995) and tissues of human Group II phospholipase A2-transgenic mice (Nevalainen et al. 1997). Positive immunostaining was found in the Paneth cells of the human intestinal mucosa. In the human Group II phospholipase A2 transgenic mice, the same pattern of distribution of PLA2 was seen in various tissues as with the anti-human Group II phospholipase A2 antibody (data not shown).

Distribution of Group II Phospholipase A2 in Rat Tissues

Tissues from Sprague–Dawley rats of both sexes were studied by immunohistochemistry for the presence of PLA2 protein and by Northern and in situ hybridizations for the mRNA of PLA2. The results are summarized in Table 1.

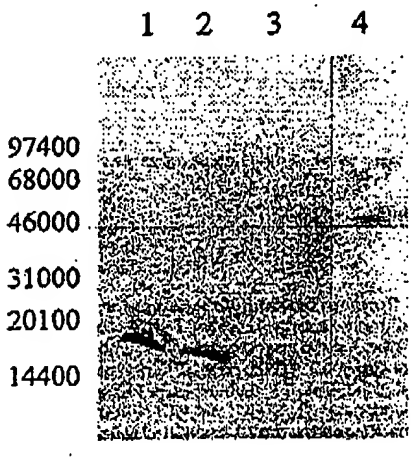

Figure 4 Western blotting with anti-rat PLA2 antibody. Lane 1, 10 ng; Lane 2, 5 ng; Lane 3, 1 ng of recombinant group II PLA2; and Lane 4, extract of rat ileum.

Gastrointestinal Tract. The Paneth cells were strongly immunoreactive in the duodenum, jejunum, ileum, and cecum. The immunoreaction was localized in the secretory granules of Paneth cells (Figure 5). Many goblet cells showed moderate immunoreaction in the small intestine and colon. No immunoreaction was found in the walls of vessels in the lamina propria or in the smooth muscle cell layer. There was no immunoreaction in the esophageal mucosa, mucosa of the glandular stomach, or pancreas. Periportal and centrilobular hepatocytes were lightly stained, whereas other cell types in the liver were unstained.

Spleen. There were large multinuclear cells with strong immunoreaction in the red pulp area of the spleen (Figure 6). The same cells were immunoreactive for

Table 1 Immunohistochemical localization and Northern and in situ hybridization of group II phospholipase A2 in rat tissues

Organ/Cell type	Immunohisto-chemistry	Northern hybridization	In situ hybridization
Liver/hepatocyte	+	–	–
Spleen/megakaryocyte	++	++	–
Lymph node	–	ns	–
Pancreas	–	–	–
Esophagus	–	+	–
Ventricle	–	+	–
Small intestine/Paneth cell	+++	+++	+++
Cecum/Paneth cell	++	+++	–
Colon/goblet cell	+	++	–
Rectum	+	ns	–
Trachea	+	ns	–
Lung	–	++	–
Heart	–	+	–
Aorta	–	+	–
Skeletal muscle	–	–	–
Cartilage/chondrocyte	++	ns	–
Thymus	–	++	–
Parotid gland	–	–	–
Lacrimal gland	–	–	–
Harderian gland	–	ns	–
Eye	–	ns	–
Brain	–	–	–
Skin	–	+	–
Adrenal gland	–	–	–
Kidney	–	–	–
Urinary bladder	–	++	–
Ureter	–	ns	–
Testis	–	–	–
Epididymis	–	–	–
Prostate	–	–	–
Seminal vesicle	–	–	–
Ovary	–	ns	–
Fallopian tube	–	ns	–
Uterus	–	ns	–

–, no signal; +, weak signal or signal in occasional cells only; ++, moderate signal; +++, strong signal; ns, tissues not studied.

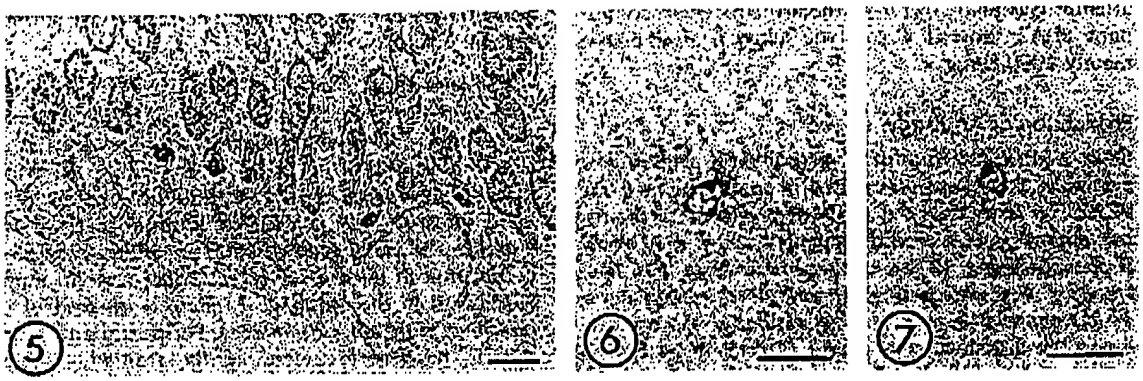

Figure 5 Immunoreaction for rat Group II phospholipase A2 in the the small intestinal mucosa. The reaction is localized in the secretory granules of the Paneth cells. Anti-rat PLA2 antiserum 1:20,000, avidin-biotin-peroxidase complex (ABC). Bar = 50 µm.

Figure 6 Immunoreaction for PLA2 in megakaryocytes in the spleen. Anti-rat PLA2 antiserum 1:5000, ABC. Bar = 50 µm.

Figure 7 Immunoreaction for von Willebrand factor in megakaryocytes of the spleen. Anti-von Willebrand factor 1:1000, ABC. Bar = 50 µm.

von Willebrand factor (Figure 7), suggesting that these cells were megakaryocytes.

Northern hybridization showed approximately an 800-base transcript in the esophagus, glandular stomach, duodenum, jejunum, ileum, cecum, colon, and spleen (Figure 8). The largest amount of PLA2 transcript was found in the ileum. By in situ hybridization, mRNA of PLA2 was found in the Paneth cells only (Figure 9). In situ hybridization with the control riboprobe gave negative results in the intestinal mucosa (Figure 10) and all other tissues studied.

Respiratory Organs and Cartilage. There was moderate immunoreaction in the chondrocytes and the matrix of the cartilage of the trachea and in the main bronchus. Costal cartilage was also immunostained. There was no immunoreaction in the other cell types of the lung. Northern blotting detected mRNA of PLA2 in the lung, but in situ hybridization gave negative results.

Heart and Circulatory System. There was no immunoreaction in the myocytes of the heart or the aortic wall. Northern blotting detected mRNA of PLA2 in the heart, whereas in situ hybridization gave negative results.

Urinary and Reproductive Organs. Neither immunoreaction or Northern blotting signal was observed in the kidney, prostate, seminal vesicle, testis, epididymis, ovary, Fallopian tube, and uterus. Urinary bladder gave a signal in Northern blotting but no immunoreactive material was observed.

Neural, Muscle, and Lymphoid tissues. Neural tissue and skeletal muscle were devoid of immunoreaction and Northern blotting signal. Neither lymph nodes nor adipose tissue contained immunoreactive material. Northern blotting gave negative results in the latter tissues as well.

Discussion

In this study we expressed rat Group II phospholipase A2 in *E. coli* and produced antibodies against the recombinant protein. Furthermore, we investigated the distribution of PLA2 protein and its mRNA in various rat organs and cell types.

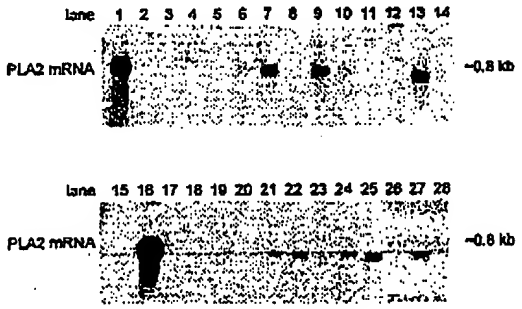

Figure 8 Northern blotting of rat tissues for the mRNA of Group II phospholipase A2. Lane 1, cecum; Lane 2, liver; Lane 3, prostate; Lane 4, testis; Lane 5, pancreas; Lane 6, ventricle; Lane 7, lung; Lane 8, kidney; Lane 9, spleen; Lane 10, skin; Lane 11, cerebellum; Lane 12, adrenal gland; Lane 13, jejunum; Lane 14, lacrimal gland; Lane 15, adrenal gland; Lane 16, ileum; Lane 17, epididymis; Lane 18, parotid gland; Lane 19, heart; Lane 20, seminal vesicle; Lane 21, esophagus; Lane 22, duodenum; Lane 23, aorta; Lane 24, thymus; Lane 25, urinary bladder; Lane 26, skeletal musde; Lane 27, colon; and Lane 28, brain. Each lane was loaded with 10 µg RNA and the autoradiograph was exposed for 4 days.

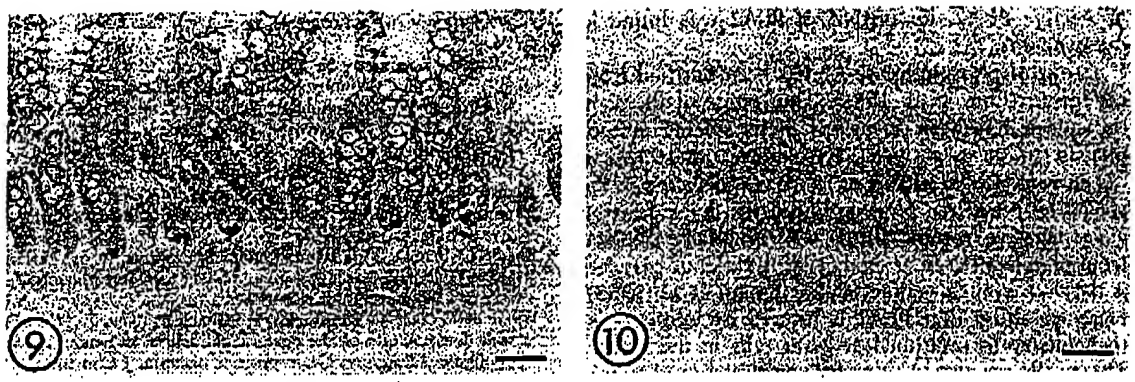

Figure 9 Positive in situ hybridization for the mRNA of Group II phospholipase A2 in rat ileum. The signal is localized in the Paneth cells. Anti-sense riboprobe. Bar = 50 μm.

Figure 10 Negative in situ hybridization for the mRNA of Group II phospholipase A2 in rat ileum. Control (sense) riboprobe. Bar = 50 μm.

The recombinant protein was generated by expressing the protein-coding sequence of rat PLA2 with an attached C-terminal histidine tag for rapid purification. Rat PLA2 cDNA was cloned and sequenced earlier (Ishizaki et al. 1989). It consists of 758 nucleotides coding for a 146-amino-acid protein. Rat group II PLA2 protein contains a 21-amino-acid N-terminal signal sequence, and the N-terminus of secreted PLA2 is serine at position 22 (Ono et al. 1988). We sequenced six N-terminal amino acids in the current recombinant rat PLA2 and found that, in 83% of the protein, the first amino acid was serine, as in native rat PLA2 (Ono et al. 1988). The recombinant PLA2 produced in the present study was catalytically active, which might be due to partial refolding caused by oxidation during the dialysis against water.

In this study we investigated the distribution of PLA2 and its mRNA in rat tissues. We used immunohistochemistry to detect the PLA2 protein and Northern and in situ hybridizations to detect the mRNA. As far as we are aware, the present study contains the first data on in situ hybridization of mRNA of Group II phospholipase A2 in the rat. We found the mRNA in the Paneth cells of the intestinal mucosa. Despite positive results in Northern blotting, no in situ hybridization signals were detected in other cell types. This finding indicates that the level of the gene expression of PLA2 in rat tissues may be below the detection limit of the current methods of tissue preservation and in situ hybridization.

Despite the high homology between human and rat Group II phospholipase A2 proteins, we have not previously found any crossreactivity between human anti-Group II phospholipase A2 antibody and rat PLA2 (unpublished data). In this study, we show crossreactivity between the anti-rat PLA2 antiserum and human Group II phospholipase A2 in immunohistochemistry. The Paneth cells of the human intestinal mucosa were immunostained with the anti-rat PLA2 antisera, and the latter crossreacted with the tissues containing human PLA2 in human Group II phospholipase A2-transgenic mice (Nevalainen et al. 1997).

Earlier, the distribution of PLA2 was studied in the gastrointestinal tract of humans (Kiyohara et al. 1992; Nevalainen et al. 1995) and rats (Senegas-Balas et al. 1984). The results have been divergent, probably due to the use of different antibodies. Synthesis of PLA2 was demonstrated in the Paneth cells of the intestinal mucosa by immunohistochemistry (Senegas-Balas et al. 1984; Kiyohara et al. 1992; Nevalainen et al. 1995) and by in situ hybridization (Nevalainen et al. 1995). In the present study, we found PLA2 protein and mRNA in the Paneth cells of rat intestine. Kiyohara et al. (1992) found PLA2 in the epithelial cells of the esophagus, stomach, duodenum, small intestine, and ascending colon. We found immunoreactive material in the goblet cells of the small intestine and colon but not in the epithelial cells of the esophagus or glandular stomach. On the other hand, we found mRNA of PLA2 by Northern blotting in the esophagus, glandular stomach, and colon.

It has been proposed that Group II phospholipase A2 has antibacterial effects in the gastrointestinal tract. PLA2 appears to be involved, together with other enzymes (e.g., lysozyme) and cryptidins in the protection of the small intestinal crypts against microbial invasion (Harwig et al. 1995). Kiyohara and others (1992) found PLA2 also in pancreatic acinar cells, Kupffer cells of the liver, and hepatocytes. Non-neoplastic hepatocytes of a patient suffering from an epithelioid hemangioendothelioma of the liver were found to express PLA2 (Nevalainen et al. 1996). In the current study, we found a slight immunostaining of some hepatocytes but no mRNA in the liver. Neither immu-

noreactive PLA2 nor mRNA of PLA2 was found in the pancreas.

Group II phospholipase A2 has been purified from rat spleen (Ono et al. 1988). The source of PLA2 in the spleen has been suggested to be splenic macrophages (Inada et al. 1991; Kiyohara et al. 1992). In the current study, mRNA of PLA2 was detected in the spleen by Northern blotting but in situ hybridization gave negative results. PLA2 was originally purified from platelets (Kramer et al. 1989). PLA2 of platelets is derived from megakaryocytes (Emadi et al. 1998). Here we show that PLA2 is located by immunohistochemistry in multinuclear giant cells in the spleen. These cells are also positive for von Willebrand factor, suggesting that they are megakaryocytes rather than monocyte macrophage-derived cells.

Recently, PLA2 was cloned from rat heart (De Windt et al. 1997) and PLA2 gene expression was observed in the heart, isolated ventricular myocytes, and cardiac-derived fibroblast-like cells by Northern blotting. PLA2 has been localized by immunohistochemistry in the ventricular myocytes of rat heart (Kriegsmann et al. 1993). However, in the current study, we did not find immunoreactive PLA2 in ventricular myocytes, although we detected mRNA of PLA2 in the heart by Northern blotting. This may be because there are very small amounts of the enzyme in myocytes.

Synovial fluid, chondrocytes, and the matrix of cartilage contain large amounts of PLA2 in the human (Nevalainen et al. 1993). Here, we found PLA2 in extra-articular cartilage (costal and tracheal cartilage). However, mRNA of PLA2 was not detected in chondrocytes by in situ hybridization.

In summary, we describe the production of large amounts of recombinant rat Group II phospholipase A2 and polyclonal rabbit antibodies against PLA2. Immunohistochemical, Northern blotting, and in situ hybridization analyses allowed a detailed characterization of the distribution of PLA2 in rat tissues. These methods provide useful tools for studies on the role of PLA2 in various experimental disease models.

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ARTICLE

Cellular Localization of Group IIA Phospholipase A_2 in Rats

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SUMMARY It has been known that group II phospholipase A_2 (PLA_2) mRNA and protein are present in the homogenates of the spleen, lung, liver, and kidney in normal rats, but the cellular origin of this enzyme has not been yet identified. At present, five subtypes of group II PLA_2 have been identified in mammals. Antibodies or mRNA probes previously used for detecting group II PLA_2 need to be evaluated to identify the subtypes of group II PLA_2. In this study we tried to identify group IIA PLA_2-producing cells in normal rat tissues by in situ hybridization (ISH) using an almost full-length RNA probe for rat group IIA enzyme. Group IIA PLA_2 mRNA was detected in megakaryocytes in the spleen and Paneth cells in the intestine by ISH. These cells were also immunopositive for an antibody raised against group IIA PLA_2 isolated from rat platelets. Group IIA PLA_2 mRNA-positive cells were not detected in lung, liver, kidney, and pancreas. Under normal conditions, group IIA PLA_2-producing cells are splenic megakaryocytes and intestinal Paneth cells in rats.

(J Histochem Cytochem 49:777–782)

KEY WORDS
group IIA phospholipase A_2
immunohistochemistry
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rat
spleen
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PHOSPHOLIPASE A_2 (PLA_2) comprises a diverse family of enzymes that catalyzes the hydrolysis of glycerophospholipids at the sn-2 position to produce free fatty acids and lysophospholipids (Tischfield 1997; Valentin et al. 1999). At present, mammalian secretory PLA_2s are classified into groups I, II, V, and X. Group II PLA_2 has been further classified into five subtypes (Type IIA, IIC, IID, IIE, and IIF) on the basis of their primary structures. Group II PLA_2 is one of the key enzymes in the process of inflammation that regulates the synthesis of arachidonic acid and lysophospholipids (Nevalainen 1993; Nevalainen et al. 1993). High plasma levels of group II PLA_2 have been observed in patients with septic shock, multiple injuries, rheumatoid arthritis, and acute pancreatitis, and a specific inhibitor of group II PLA_2 improved the mortality in experimental pancreatitis (Yoshikawa et al. 1999).

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Group II PLA_2 mRNA and protein were detected in homogenates of the spleen, lung, liver, and kidney of normal rats (Murakami et al. 1988; Ishizaki et al. 1989; Inada et al. 1991a; Hara et al. 1995; De Windt et al. 1997), but the cellular origins of this enzyme have not been yet identified. Although PLA_2, referred to as group II PLA_2 in previous reports, was assumed to be a group IIA subtype, in the light of recent findings on the diversity of this group of enzymes, characteristics of antibodies or mRNA probes need to be specified. In the present study, therefore, we prepared an almost full-length RNA probe for group IIA PLA_2 mRNA and tried to identify group IIA PLA_2-producing cells in normal rat tissues using both ISH and IHC techniques.

Materials and Methods

Preparation of Rats

Male Wistar rats (180–220 g) were anesthetized with an IP injection of pentobarbital sodium (50 mg/kg) and transcardially perfused with Tyrode's solution and then with 4% paraformaldehyde in 0.01 M PBS, pH 7.4. The jejunum, spleen, lung, liver, kidney, and pancreas were removed and postfixed in the same fixative for 3 hr. They were then im-

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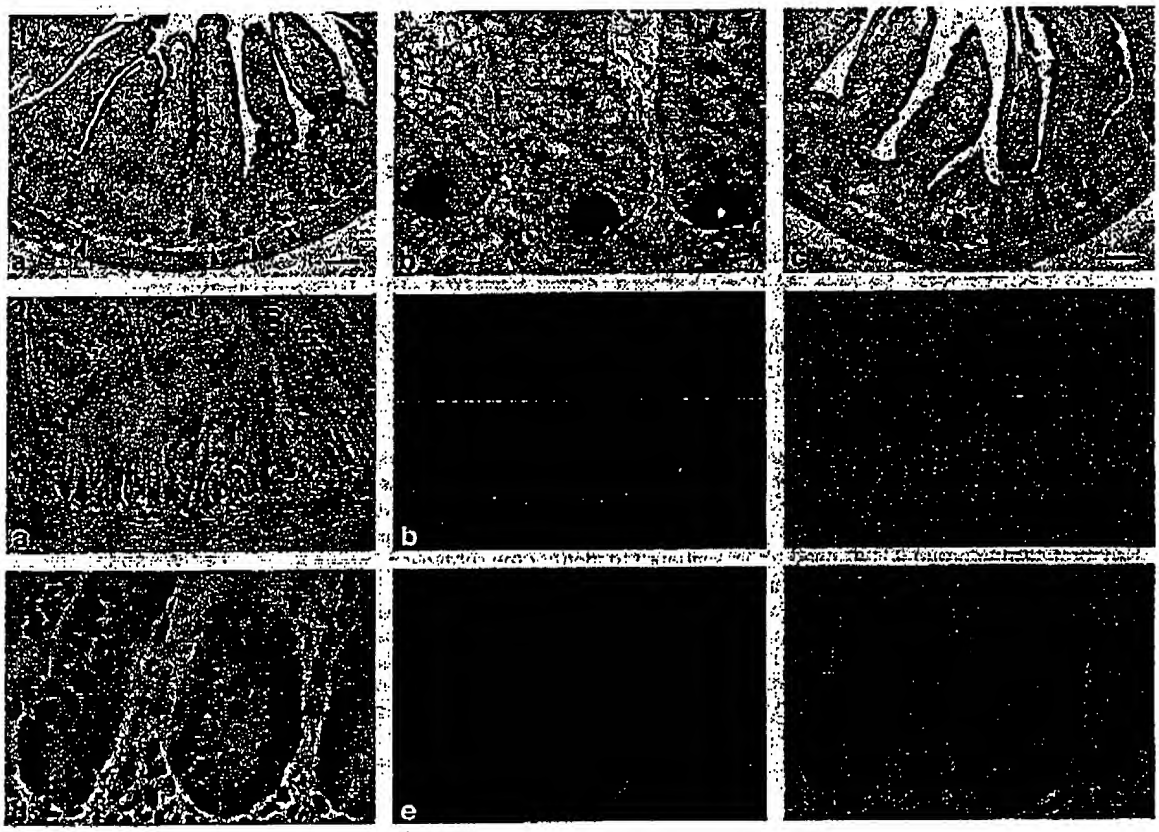


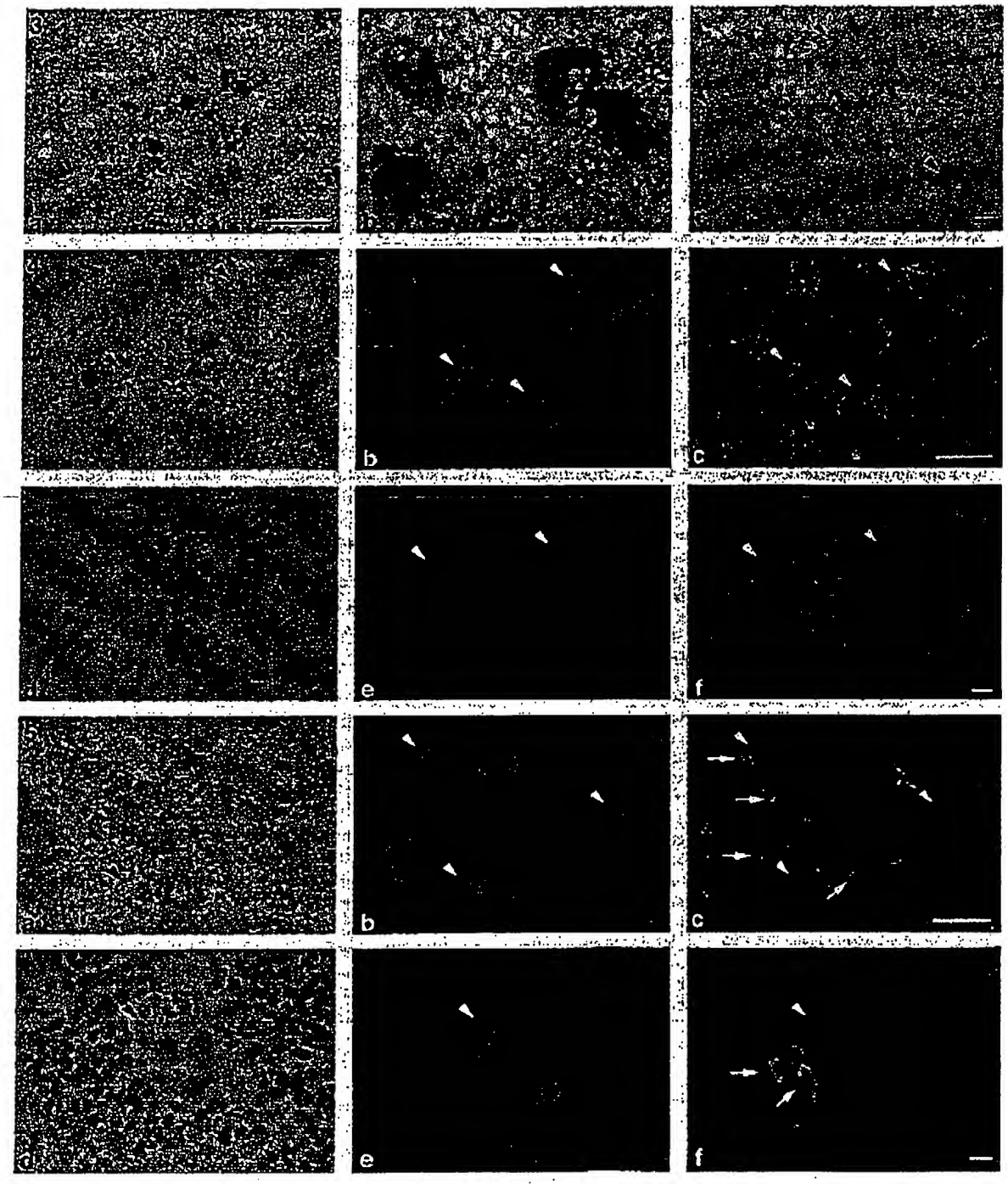
Figure 1 Group IIA PLA_2 mRNA distribution in the rat jejunum. ISH with antisense probe for group IIA PLA_2 (a,b) and negative control with sense probe (c). The signals of group IIA PLA_2 mRNA were observed in the bottom of crypts (arrowheads in a). No signal was seen in the crypts in the negative control. Staining of the epithelial cells in the negative control indicated that it was due to the intrinsic alkaline phosphatase (c). Bars: a,c = 100 μm; b = 10 μm.

Figure 2 Group IIA PLA_2-positive cells in the rat jejunum. Hematoxylin staining (a,d) and immunofluorescent staining for group IIA PLA_2 (b,e). (c) Image synthesized from a and b. (f) Image synthesized from d and e. In the mucosa of the jejunum, group IIA PLA_2 immunoreactivity was observed in the bottom of crypts (a–c). These cells were pyramidal with a round or ovoid nucleus. Group IIA PLA_2-positive immunoreactivity was localized in the luminal part of cytoplasm (d–f). Bars: c = 100 μm; f = 10 μm.

Figure 3 Group IIA PLA_2 mRNA expression in the rat spleen. ISH with antisense probe for group IIA PLA_2 (a,b) and negative control with sense probe (c). Group IIA PLA_2 mRNA was identified in the cytoplasm of large cells (30–40 μm) with multilobular nucleus in the red pulp (a,b). In negative control, signals of these cells were as weak as background (arrowheads in c). Group IIA PLA_2 mRNA was not observed in any other cells in the spleen. Bars: a = 100 μm; c = 10 μm.

Figure 4 Group IIA PLA_2-positive megakaryocytes in rat spleen. Hematoxylin staining (a,d) and double labeling of group IIA PLA_2 (b,e) and fibrinogen (c,f). Group IIA PLA_2 immunoreactivity was observed in large (30–40 μm) cells with multilobular nuclei in the red pulp (arrowheads in a and b). The immunoreactivity was diffusely present in the cytoplasm of these cells (arrowheads in d and e). The double immunolabeling of group IIA PLA_2 and fibrinogen showed that all of these group IIA PLA_2-positive cells had fibrinogen immunoreactivity (arrowheads in c and f). These cells were consistent with megakaryocytes. Bars: c = 100 μm; f = 10 μm.

Figure 5 Group IIA PLA_2 and macrophages in rat spleen. Hematoxylin staining (a,d) and double labeling of group IIA PLA_2 (b,e) and ED1 (c,f). Large (about 10 μm) mononuclear cytoplasm-rich cells in the red pulp were positive for ED1 (arrows in c and f). These ED1 positive cells were consistent with macrophages. Group IIA PLA_2 (arrowheads) and ED1 (arrows) were not co-localized in any cells. Only megakaryocytes showed group IIA PLA_2 immunoreactivity. Bars: c = 100 μm; f = 10 μm.


a
b
c
d
e
f
a
b
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e
f

mersed in a series of cold sucrose solutions (10%, 20%, 30% sucrose in PBS). Ten-μm-thick sections were cut on a cryostat microtome (Bright; Huntingdon, UK) and mounted on silanized slides (DAKO; Kyoto, Japan). The sections were dried on a hotplate at 37C and then kept frozen until use.

Digoxigenin-labeled RNA Probe

We used a 0.7 kilobase pair fragment of digoxigenin-labeled RNA probe in the present study. This RNA probe corresponds to bp 76–748 of group IIA PLA_2 mRNA, which covers about 90% of the whole mRNA. The NheI/EcoRI 0.7-kb pair fragment of rat group II PLA_2 cDNA was excised from its full-length cDNA (Ishizaki et al. 1989) and subcloned into the XbaI/EcoRI sites of the pGEM-3Z. Antisense digoxigenin-labeled RNA probes were prepared using digoxigenin-11-UTP and T7 RNA polymerase to transcribe the group II PLA_2 insert cDNA. Sense strand RNA probes were generated using SP6 RNA polymerase.

In Situ Hybridization

The sections were incubated in 0.3% Triton X-100 in PBS for 5 min and immersed in 10 μg/ml proteinase K (Boehringer Mannheim Biochemical; Indianapolis, IN) in PBS for partial proteolysis for 10 min at 37C. They were postfixed in 4% paraformaldehyde in PBS for 10 min and immersed in a solution containing 0.25% acetic acid anhydride (Nippon Gene; Toyama, Japan), 0.1 M triethanolamine for 20 min at room temperature (RT). They were then prehybridized in 50% formamide in 2 × SSC for 30 min at 42C. Hybridization was done with digoxigenin-labeled RNA antisense probe diluted to 1 μg/ml in hybridization buffer in a moist chamber for 16 hr at 42C. For negative control, digoxigenin-labeled RNA sense probe was hybridized to a section adjacent to the test section. After hybridization, the slides were washed in 50% formamide in 2 × SSC for 60 min at 42C and treated with 20 μg/ml RNase A in 0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) for 30 min at 37C. They were rinsed in the same buffer at 37C for 30 min, then in 0.1 × SSC for 60 min at 42C, and finally in PBS for 1 min at RT. For immunological detection of digoxigenin, the slides were preblocked in 1% skim milk fraction in PBS for 30 min at RT and then incubated with alkaline phosphatase-conjugated polyclonal anti-digoxigenin Fab fragments (Boehringer Mannheim) (1:500 dilution) for 90 min at RT. After incubation, the slides were washed in PBS and developed in a solution of nitroblue tetrazolium and X-phosphate for 16 hr at RT. The color reaction was stopped using 1 mM EDTA, 10 mM Tris-HCl. The sections were mounted in Vectashield (Vector Laboratories; Burlingame, CA).

Immunohistochemistry

Rabbit anti-rat group II PLA_2 IgG raised against purified PLA_2 released from thrombin-stimulated rat platelets was used for histochemistry. The slides were rinsed with PBS and nonspecific binding was blocked in 1% skim milk in PBS. Sections were incubated with rabbit anti-rat group II PLA_2 antibody. (Nakano et al. 1990) (1:2000 dilution) in a moist chamber overnight at RT, followed by Alexa546-conjugated goat anti-rabbit IgG (Molecular Probes; Eugene, OR) (1:100 dilution). Double-labeling studies were performed to characterize the group II PLA_2-positive cells. For the second staining sequence, mouse anti-rat ED1 monoclonal antibody (Chemicon International; Temecula, CA) (1:400 dilution) or goat anti-rat fibrinogen antibody (ICN Pharmaceuticals; Aurora, OH) (1:1000 dilution) was applied as second primary antibody. Then sections were incubated with biotinylated secondary antibody (Vector) and streptavidin–FITC (Amersham Pharmacia Biotech; Tokyo, Japan). The sections were mounted with Vectashield. The sections were examined with an Olympus epifluorescence microscope (Provis AX80; Olympus, Tokyo, Japan). To check the specificity of the immunolabeling, the same immunostaining procedure was followed except that preimmune rabbit or goat serum was applied as the primary antibody. No appreciably stained structures were observed.

Results

Jejunum

In the jejunum, group IIA PLA_2 mRNA-positive cells were present in the bottom of crypts (Figures 1a and 1b). No significant signal was seen in the crypts in the negative control. Staining of the epithelial cells in the negative control indicated that it was due to the intrinsic alkaline phosphatase (Figure 1c). Group IIA PLA_2 immunoreactivity was also observed in the bottom of crypts (Figures 2a–2c). These cells were pyramidal, with a round or ovoid nucleus (Figures 2d–2f). These characteristic features indicated that they were Paneth cells.

Spleen

ISH revealed that signals of group IIA PLA_2 mRNA were observed in large cells (~30–40 μm) with multilobular nuclei in the red pulp (Figures 3a and 3b). Group IIA PLA_2-positive cells were roughly spherical but often had blunt, irregular pseudopods on their surfaces. Their nuclei were extraordinarily elaborate, with multiple lobes of various sizes. The double-labeling study demonstrated that group IIA PLA_2-positive cells were also positive for fibrinogen (Figures 4a–4f). These morphological and immunohistochemical findings indicated that group IIA PLA_2-positive cells in the spleen were megakaryocytes. Neither group IIA PLA_2 mRNA nor immunoreactivity was observed in other types of cells in the spleen.

The ED-1 immunohistological study revealed that ED1-positive cells were found in the red pulp. These cells were ~10 μm in diameter, mononuclear, and rich in cytoplasm. Therefore, these ED1-positive cells in the red pulp appeared to be macrophages. A double-immunolabeling of group IIA PLA_2 and ED1 revealed that group IIA PLA_2 and ED1 were never co-localized in any cells. Thus, group IIA PLA_2-positive cells were completely separated from ED1 positive cells (Figures 5a–5f). These results indicated that megakaryocytes, but not macrophages, contained group IIA PLA_2.

Other Organs

Group IIA PLA_2 mRNA and immunoreactivity were not detected in any cells of the lung, liver, kidney, and pancreas.

Discussion

In this study, using a specific RNA probe and an antibody for rat group IIA PLA_2, we demonstrated that group IIA PLA_2-producing cells were megakaryocytes in the spleen and Paneth cells in the small intestine in normal rats. The antibody used in the present study was raised against group IIA PLA_2 isolated from the rat platelet (Nakano et al. 1990). Although crossreactivities of this antibody with other groups of PLA_2s were not examined, only those cells that expressed group IIA mRNA showed a positive reaction with this antibody. It has been reported that group V PLA_2 produced by a murine P388D1 macrophage has crossreactivity with the antibody raised against human synovial PLA_2, a group IIA enzyme (Balboa et al. 1996). The amino acid sequence identities of mouse IIA PLA_2 with other types of PLA_2 are 31–51% (Valentin et al. 1999). The overall amino acid identity of rat group IIA and group V PLA_2 was reported to be 42.5% (Chen et al. 1994). Because the RNA probe used in the present study covers about 90% of the total mRNA of group IIA PLA_2, it is unlikely that this probe detects mRNAs of other groups of PLA_2.

IHC of smeared bone marrow cells indicated that megakaryocytes produced group II PLA_2 (Murakami et al. 1990). Inada et al. (1991b) tried to identify the cellular origin of group II PLA_2 by separating spleen cells on a density gradient. They found that about one third of the cells in the monocyte/macrophage fraction were immunopositive for group II PLA_2. Although about 70% of cells in the monocyte/macrophage fraction were of macrophage nature, i.e., immunopositive for ED1, it was not shown that megakaryocytes were not included in the remaining 30% of cells in this fraction. To solve this problem, we performed a double staining of the spleen with both antibodies and found that no group II PLA_2 protein was present in the macrophages of the spleen.

The concentration of group II PLA_2 was very low in human spleen (Murakami et al. 1988; Nevalainen and Haapanen 1993). Immunohistochemical study failed to detect any group II PLA_2 immunopositive cells in the human spleen (Kallajoki and Nevalainen 1997). The spleen of rodents, unlike that of human, is rich in megakaryocytes. This can explain the quantitative difference of group II PLA_2 in the spleen between rat and human.

Northern blotting analysis (Ishizaki et al. 1989) indicated the presence of group II PLA_2 mRNA in normal rat small intestine, but the cellular origin was not identified. In the human intestine, group II PLA_2 was detected in Paneth cells by both IHC and ISH (Nevalainen et al. 1995). The present study confirmed the production of group IIA PLA_2 in Paneth cells in rats. The function of PLA_2 in Paneth cells is not fully understood. It is believed that they are involved in the host defense because they produce lysozymes and PLA_2, both of which have antibacterial properties (Sandow and Whitehead 1979; Wright et al. 1990). Recently, it was shown that group IIA PLA_2-deficient mice were susceptible to multiple intestinal adenomas (MacPhee et al. 1995). Therefore, group IIA PLA_2 may modulate cell proliferation directly by acting on tumor cells or indirectly by removing potentially harmful dietary fatty acids or bacteria.

Because group II PLA_2-specific activities, protein, or mRNA were present in the homogenate of the normal rat liver (Inada et al. 1991b; Dong et al. 1997), kidney (Hara et al. 1995), lung (Murakami et al. 1988; Ishizaki et al. 1989), and pancreas (Lauritzen et al. 1994), we tried to identify group IIA PLA_2-producing cells in these tissues by IHC and ISH. However, we could not detect group IIA PLA_2 protein and mRNA in normal rat liver, kidney, lung, and pancreas. One explanation for this discrepancy is that the amounts of group II PLA_2 protein and mRNA in these organs were much less than those of the spleen (Murakami et al. 1990; De Windt et al. 1997). Therefore, the expression of group IIA PLA_2 in these tissues may be too low to be detected by our methods. Another possibility is that group IIA PLA_2 protein or mRNA in the megakaryocytes present in the circulation (Kaufman et al. 1965) may have been detected in the previous studies. In this study, we removed the intravascular cellular elements by perfusing blood vessels with Tyrode's solution. In view of the diversity of PLA_2s, further studies are necessary to identify the cellular origin of each subtype of this enzyme.

In conclusion, under normal conditions group IIA PLA_2-producing cells are splenic megakaryocytes and intestinal Paneth cells in rats. The induction of group IIA PLA_2 in other types of cells under inflammatory conditions awaits further studies.

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